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(54) Title: AMPICILLIN AND AMOXICILLIN-BASED POLY(ANHYDRIDE-AMIDES)
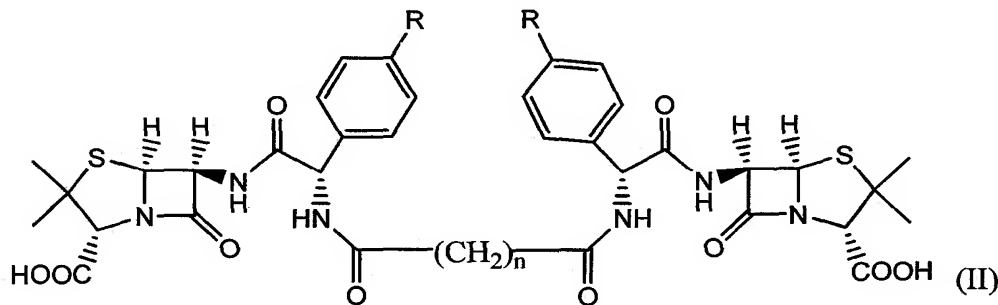
(57) Abstract: In one aspect the invention relates to an antibacterial compound of formula II: wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12; R is H or OH; or a salt thereof. In other aspects the invention also provides devices that include the compound as well as methods for producing an antibacterial effect with the compound or its salt.

AMPICILLIN AND AMOXICILLIN-BASED POLY(ANHYDRIDE-AMIDES)

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Government Funding

The invention described herein was made with government support under Grant Number DE 13207 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

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Related Application(s)

This patent document claims the benefit of priority of U.S. application serial No. 60/957,359, filed August 22, 2007, which application is herein incorporated by reference.

Background of the Invention

15   Bacterial infections are a safety issue with medical implants, including stainless steel materials; such infections are extremely difficult to treat due to the development of resistant biofilm networks that are not susceptible to traditional antibiotics (Patel R. Biofilms and antimicrobial resistance. *Clin Orthop Relat Res,* **2005,** *437,* 41-47). Such infections often lead to multiple revision surgeries,

20   and in some cases, death (Campoccia D, et al., *Biomaterials,* **2006,** *27,* 2331-2339).

Typically, bacterial infections are systemically treated with a course of antibiotics. However, systemic treatments expose the entire body to the drug such that the implant site may not receive the dosage necessary to kill the

25   bacteria (Burrows LL, Khoury AE. Infection of medical devices. In: Wnek GE, Bowlin GL., editor. Encyclopedia of Biomaterials and Biomedical Engineering. New York: Marcel Dekkar; 2004.839-848). Often, this lowered dose results in the development of resistant bacteria, and ultimately biofilm formation (Hanssen AD, et al., *Clin Orthop Relat Res,* **2005,** *437,* 111-114).

30   Localized antibiotic therapy can also be used to treat bacterial infections associated with medical implants. The most commonly studied localized antibiotic therapy is the physical admixture of antimicrobials within polymer matrices to treat bacterial infections (see von Eiff C, et al., *Int J Artif Organs,*

2005, *28*, 1146-56; Garvin K, Feschuk, *Clin Orthop Relat Res*, **2005**, *437*, 105-
110; Gollwitzer H, et al., *J Antimicrob Chemother*, **2003**, *51*, 585-591 and
Sharkawi T, et al., *J Bioact and Compat Pol*, **2005**, *20*, 153-168). Polymer
coatings for medical device applications have been characterized throughout the
literature (see Roosjen A, et al., *J Biomed Mater Res Part B*, *Appl Biomater*,
2005, *73B*, 347-354; Tedjo C, et al., *J Biomed Mater Res*, **2007**, *82A*, 479-491;
Song R, et al., *Polymer*, **2005**, *46*, 1643-1652; Sarisuta N, et al., *Int J Pharm*,
1999, 186:109-118; and Ruggeri V, et al., *J Biomed Mater Res*, **2007**, *81A*, 287-
298).

In spite of the above reports, there is currently a need for agents and
methods that are useful for treating bacterial infections. For example, there is a
need for agents and methods that are useful for treating bacterial infections
associated with medical implants (*e.g.*, orthopedic implants).

<u>Summary of the Invention</u>

In one embodiment, the present invention provides compounds that have
antibacterial properties. Accordingly there is provided a compound of the
invention which is a compound of formula I:

(I)
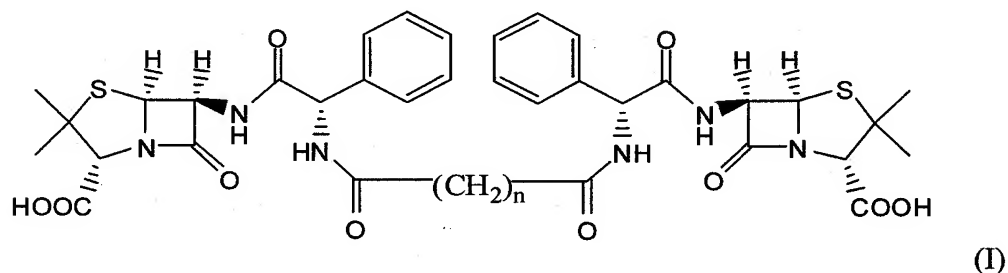
wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12; or a salt thereof.

Certain embodiments of the invention provide a compound of formula II:

(II)

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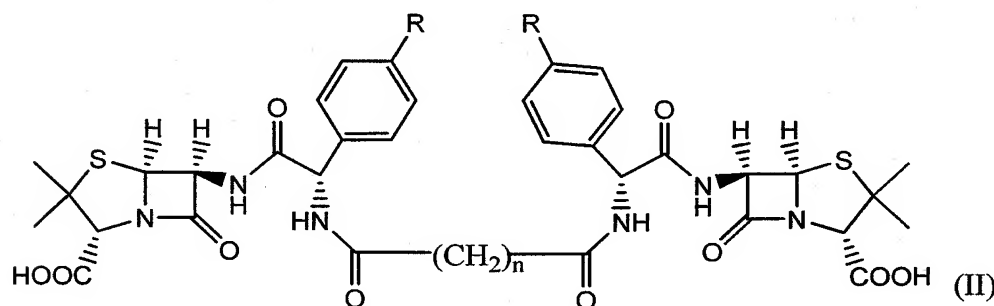
wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12; and

each R is independently H or OH;

or a salt thereof.

In certain embodiments, each R is OH.

In certain embodiments, each R is H.

In certain embodiments, one R is H and one R is OH.

In certain embodiments, n is 4, 5, 6, 7 or 8.

In certain embodiments, n is 6, 7 or 8.

In certain embodiments, n is 8.

In one embodiment the invention also provides a pharmaceutical composition comprising a compound of the invention and a pharmaceutically acceptable diluent or carrier.

In one embodiment the invention also provides a medical device having a coating that comprises a compound of the invention.

In one embodiment the invention also provides a medical device comprising a compound of the invention.

In certain embodiments, the medical device is an orthopedic implant.

In certain embodiments, the medical device is a knee joint or a hip joint.

In one embodiment the invention also provides a method to promote an anti-bacterial effect in an animal comprising administering a compound of the invention to the animal.

In certain embodiments, the antibacterial effect is against gram negative and/or gram positive bacteria.

In one embodiment the invention also provides a method to promote an antibacterial effect comprising contacting bacteria (e.g., *in vivo* or *in vitro*) with a compound of the invention.

In one embodiment the invention also provides a method to reduce biofilm formation on a surface comprising contacting the surface with a compound of the invention.

In certain embodiments, the compound of the invention is incorporated into a coating that is on the surface.

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In one embodiment the invention also provides a compound of the invention for use in medical therapy.

In one embodiment the invention also provides the use of a compound of the invention for the manufacture of a medicament for producing an anti-

5   bacterial effect in a mammal.

In one embodiment the invention also provides a compound of the invention, for the prophylactic or therapeutic treatment of a bacterial infection.

The invention also provides processes and intermediates disclosed herein that are useful for preparing compounds of formula (I) or (II) or salts thereof.

10   The compounds, compositions and methods of the invention are useful for preventing biofilm formation and for treating antibiotic resistant bacterial infections, such as those infections associated with medical implants.

Detailed Description

The use of polymeric materials for biological applications such as medical

15   devices (*e.g.*, artificial organs, wound dressings, sutures, pacemakers, intravascular catheters, contact lenses, orthopedic implants) and drug delivery systems has evoked increasing interest. Numerous polymeric biomaterials are implanted each year in humans, yet bacterial infections associated with these medical implants remain a significant issue. For example, nearly half of the two

20   million nosocomial infections occurring in the United States are associated with medical implants. The presence of a foreign body, such as a prosthetic implant, provides a site for microbial adhesion and subsequent colonization, often leading to device-related infections. Current clinical therapies to prevent biomaterial-related infections involve the lengthy administration of antibiotics at high

25   systemic concentrations. Unfortunately, repeated failures of this therapeutic approach require device removal to ensure eradication of the infection and to avoid relapse.

As described herein, degradable biomaterials were designed to release antimicrobials upon polymer degradation to locally prevent infections. This

30   work describes the synthesis of biodegradable antimicrobial-based polymers in which the antimicrobial drugs ampicillin and amoxicillin are chemically incorporated into the polymeric backbone by anhydride and amide bonds, rather

than attached as a side group or physically admixed. Antibacterial properties of the polymers were examined using gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus aureus*) bacterial strains. *E. coli* is the major cause of infections in biliary stents and urinary catheters, and *S. aureus* is responsible for

5   infections associated with dental and orthopedic implants and fixation devices.

Antibiotic molecules (up to nearly 82% by weight) were incorporated into a polymeric backbone. The polymers were prepared under mild conditions using solution polymerization methods. This milder polymerization method was chosen over typical melt-condensation processes because of the thermal

10   instability of the antibiotics and polymer precursors. No glass transition temperatures (T_g) were observed for the polymers in the measured range from −10 °C up to polymer decomposition (~ 200 °C). Notably, the antimicrobial-based polymers displayed enhanced solubility in organic solvents relative to the antibiotics alone. This factor is significant, as it determines processability of

15   polymer into solvent-cast films, microspheres, and fibers that may be used as controlled release devices.

In vitro hydrolytic degradation and antimicrobial susceptibility assays of these materials showed that antimicrobial compounds were rapidly released and inhibited the growth of gram-negative (*E. coli*) and gram-positive (*S. aureus)*

20   bacteria. These bacteria are responsible for a wide range of medical device failures as a consequence of infection. The biodegradable antimicrobial-based polymers show promise as biomaterials that may reduce implant failure due to their antimicrobial properties.

As these polymers can be used for use as medical implants or implant

25   coatings, their cytotoxicity was evaluated *in vitro* as a mean to evaluate material biocompatibility. The antimicrobial-based polymers and their degradation products showed no cytotoxic effects when in contact with fibroblast cells. Similar fibroblast proliferation and morphology was observed with different polymer concentrations in the range 0.01-0.1 mg/mL as compared to the

30   controls. Based upon these results, the biodegradable antimicrobial-based poly(anhydride-amides) display promise as implants or coatings to reduce implant failure resulting from bacterial infections.

In cases where compounds are sufficiently basic or acidic, a salt of a compound of formula I or II can be useful as an intermediate for isolating or purifying a compound of formula I or II. Additionally, administration of a compound of formula I or II as a pharmaceutically acceptable acid or base salt may be appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids which form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartrate, succinate, benzoate, ascorbate, α-ketoglutarate, and α-glycerophosphate. Suitable inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts.

Pharmaceutically acceptable salts may be obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts of carboxylic acids can also be made.

The compounds of the invention are useful in a variety of applications where antibacterial activity is desired. Examples of such applications include, but are not limited to, medical, dental and cosmetic uses.

The compounds of the invention may be prepared in accordance with methods commonly employed in the field of synthetic chemistry. The compounds can be readily processed into pastes or solvent cast to yield films, coatings, microspheres and fibers with different geometric shapes for design of various medical implants.

The compounds of the invention can be incorporated into and/or released from medical implants such as, for example, vascular grafts and stents, bone plates, sutures, implantable sensors, implantable drug delivery devices, orthopedic devices (e.g. replacement joints such as knees and hips), and stents for tissue regeneration. As described below, the diacid compounds of formula (I) or (II) can be released by hydrolysis of a corresponding anhydride polymer. In one embodiment of the invention, the compounds of formula (I) or (II) can also be incorporated (e.g. dispersed) into the matrix of such an anhydride polymer or

another polymer, and be released from that polymer over time (e.g. upon hydrolysis of the polymer).

Compounds of the invention can also be incorporated into oral formulations and into products such as skin moisturizers, cleansers, pads,

5   plasters, lotions, creams, gels, ointments, solutions, shampoos, tanning products and lipsticks for topical application.

Useful dosages of the compounds can be determined by comparing their *in vitro* activity, and *in vivo* activity of the compounds of the invention in animal models. Additionally, useful dosages can be determined by measuring the rate of

10   hydrolysis for a given polymer under various physiological conditions. The amount of a polymer required for use in treatment will vary not only with the particular polymer selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

15   The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced administrations.

The compounds of formula I or II can be formulated as pharmaceutical

20   compositions and administered to a mammalian host, such as a human patient in a variety of forms adapted to the chosen route of administration, e.g., orally or parenterally, by intravenous, intramuscular, topical or subcutaneous routes.

Thus, the present compounds may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable vehicle such as an

25   inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches,

30   capsules, elixirs, suspensions, syrups, wafers, and the like.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin;

excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example,

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by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal,

5   and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active

10   compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional

15   desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compounds may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

20   Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and

25   additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and

30   esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions which can be used to deliver the compounds of formula I or II to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No.
5   4,820,508).

The ability of a compound of the invention to prevent biofilm formation can be determined as described hereinbelow.

The invention will now be illustrated by the following non-limiting Examples. In the event that a reference designator for a structure is assigned to
10   different structures in different Examples, the reference designator should be read in the context of the specific Example in which it is included.

Example 1

Materials

15   Ampicillin was purchased from MP Biomedicals (Solon, OH). N,N-Dimethylformamide (DMF), acetic anhydride, dichloromethane, diethyl ether, pyridine, and triethylamine were purchased from Aldrich (Milwaukee, WI). All other reagents and solvents were purchased from Fisher Scientific (Fair Lawn, NJ) and used as received. Triethylamine was dried over calcium hydride, all
20   other reagents were used without further purification.

Ampicillin-based Poly(anhydride-amide) Synthesis

Briefly, the ampicillin-based diacid (**2**) was prepared in DMF with pyridine as a base at 0°C. The polymer (**1**) was synthesized using triphosgene as a coupling agent and triethylamine as an acid acceptor at 0 °C under nitrogen.
25   The resulting polymer (**1**) was isolated by pouring over diethyl ether, drying under vacuum at room temperature, and had a $M_w = 86,000$ and PDI = 1.1.
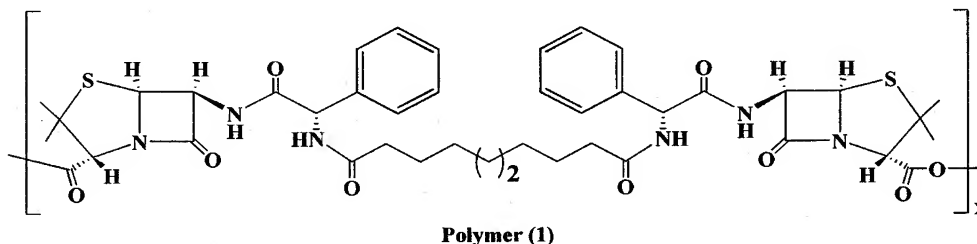
Polymer (1)

Coating Preparation and Characterization

A 5 % (w/v) ampicillin-based poly(anhydride-amide) (**1**) solution was prepared in DMF for solvent-casting. The polymer solution (150 μL) was pipetted onto 316L stainless steel coupons (McMaster-Carr, cut to 10 x 30 mm
5   by Rutgers Physics Department Machine Shop). The solvent was evaporated using one of three methods: i) ambient conditions overnight; ii) heating samples to 60 °C for two hours; and iii) heating under vacuum to 60 °C for 45 minutes. Additionally, the ampicillin-based diacid precursor was precipitated onto stainless steel substrates for comparison to the polymer coatings.

10   Peel tests were conducted using the x-cut method in American Standard Test Method (ASTM) D 3359-02 (n=5) (ASTM D 3359. West Conshohocken, PA: ASTM International; 1998). Two 10 mm X-cuts were scratched into coated coupons using a fresh razor blade. Permacel P-99 tape was adhered to the surface and peeled away at a 180° angle after 90 seconds. Each sample was
15   examined for coating removal and ranked according to the following scale: 5A-no peeling or removal; 4A-trace peeling or removal; 3A-jagged removal along incisions; 2A-jagged removal along most of the incisions; 1A-removal from most of the area of the X under the tape; and 0A-removal beyond the area of the X.

20   Sessile contact angle measurements were completed using deionized water on a model 250 Rame-Hart goniometer (Mountain Lakes, NJ) with DROPimage software. One water drop was placed on each coating and measured in real time with a camera. The contact angle was measured as the average value from five water droplets with three angle measurements taken for
25   each drop. The final contact angle value is the average of at least 15 measurements.

Digital microscope images were taken using a Keyence VHX-100 Digital Microscope (Woodcliff Lake, NJ). The coating surfaces were examined up to a magnification of 3000x to monitor coating uniformity.

30   ***In Vitro* Degradation Study**

In vitro degradation studies were conducted at the Office of Science and Engineering Labs in the Division of Chemistry and Materials Science at the Food

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and Drug Administration in Rockville, MD. Polymer-coated stainless steel

coupons were placed into individual chambers and degraded in the SOTAX USP

4 CE 7 Drug Dissolution Testing System (Allschwil, Switzerland) with a CP 7

ceramic pump, Agilent 8453 UV detection, and WinSOTAX software (see

5   Brown W. *Diss Tech,* **2005,** *12*(2), 28-30; and Beyssac E, et al., *Diss Tech,*

2005, *12*(2), 23-25). The samples were degraded at 37 °C in 0.1 M phosphate

buffer solution (PBS) pH 7.4. In-line UV detection measurements were taken

every five minutes for 4 hours and every hour subsequently at 210 nm.

Preliminary experiments were done on a Varian 400-DS USP 7 Dissolution

10   Instrument (Palo Alto, CA) under the same conditions outlined above and with

the degradation media separated by HPLC with UV detection.

Bacterial Adherence

Bacterial adherence testing was completed at Innovotech Inc. (Calgary,

Alberta, Canada) using the Biofilm Eradication Surface Testing (BEST) Assay

15   method. *Staphylococcus aureus* (ATCC 29213) was obtained from ATCC

(Manassas, VA). The coatings were exposed to the *Staphylococcus aureus*

bacteria cells at the required density for biofilm formation (10^6 colony forming

units (CFU)/mL) for 24 hours at 37 °C. Adherent bacteria cells were recovered

and quantified compared to negative uncoated controls.

20   ***In Vitro* Degradation with Bacteria Present**

Polymer-coated coupons were degraded in BHI (Brain Heart Infusion)

media (Fisher, Fair Lawn, NJ) with *S. aureus* cells for five days (cells were

maintained by Linda Rosenberg, Department of Food Science, Rutgers

University). The media was sampled at each time point, filtered through 0.45

25   μm surfactant-free cellulose acetate (SFCA) syringe filters (Nalgene, Rochester,

NY), and stored at room temperature until HPLC analysis. The samples were

transferred to HPLC vials and analyzed on a Waters 2695 Separations module

with a 2487 Dual Wavelength UV Detector and Empower 2 software. The

HPLC method was developed to separate the free ampicillin released from the

30   ampicillin diacid at 210 nm on a Gemini C18 column (150 x 4.6 mm, 5 μm,

Phenomenex, Torrance, CA). The method was a linear gradient with a mobile

phase consisting of 20 mM monobasic potassium phosphate pH 4.0 and

methanol. Five point calibration curves were generated for both the ampicillin and the ampicillin diacid with concentrations ranging from 0.005 mg/mL to 0.5 mg/mL. The degradation media was diluted in monobasic potassium phosphate, if needed, to calculate release within the calibration curve. Before HPLC

5 analysis, the samples were filtered once more through 0.45 μm PTFE syringe filters (Nalgene, Rochester, NY). Full system automation and data analysis were completed using Empower 2 software Build 2154. Complete ampicillin release was ensured by observing complete polymer degradation and calculating the total ampicillin content in the polymer.

10 *In Vitro* **Cytotoxicity**

Cytotoxicity of the polymer coatings was analyzed by culturing cells in media containing degraded polymer. Individual ampicillin-based polymer-coated stainless steel plates were placed in 50 mL of cell culture media for 48 hours. Media consisted of Dulbecco's Modified Eagle's Medium (DMEM;

15 Sigma, St. Louis, MO), 10% v/v fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), penicillin/streptomycin, and L-glutamine.

L929 mouse areolar/adipose fibroblasts (Department of Biomedical Engineering, Rutgers University, Piscataway, New Jersey) were sustained in a cell culture incubator at 37°C and an atmosphere containing 5% CO_2. Confluent

20 fibroblasts were removed from the cell culture flask via trypsinization. After media removal, trypsin (2 mg/mL, Sigma, St. Louis, MO) was placed on cells and incubated at 37°C for five minutes to allow cell detachment from the surface of the flask. Trypsin activity was ceased with addition of media and the solution centrifuged for 2 min at 2000 rpm (Thermo Electron 5682 3L GP, Franklin,

25 MA). Cells were seeded at a concentration of 100,000 cells/well in media containing polymer degradation media and analyzed after 24, 48, and 72 hours. All samples were studied in triplicate. A standard curve was generated with cells seeded at 25,000, 50,000, 100,000, and 500,000 cells/well in cell culture media without polymer, analyzed after 24 hours.

30 Imaging was performed with a fluorescent microscope (Olympus IX81, Center Valley, PA) with a 10x phase-contrast objective and cell morphology assessed by observation at 24, 48, and 72 hours.

Cell growth was quantified using Calcein AM staining as a live cell assessment every 24 hours for 72 hours. At each time point, cell culture media was removed and cells washed twice with phosphate buffered saline (PBS, pH 7.4, MP Biomedical, Aurora, OH) and refrigerated with 200 μL Calcein AM

5   stain (8 μM, Molecular Probe, Carsbad, CA) for 30 minutes at 4°C. Fluorescent intensity was quantified using a fluorescence plate reader (Cytofluor ® Series 4000, Applied Biosystems, Woodinville, CA) at 485 nm excitation and 530 nm emission. For the three days, live cell numbers were quantified against the standard curve using Microsoft Excel ®.

10   Results and Discussion

Coating Preparation and Characterization

Smooth, uniform polymer coatings containing an average of 12 mg ampicillin-based poly(anhydride-amide) were solvent-cast onto 316L stainless steel. Several parameters were investigated to produce uniform polymer

15   coatings: solvent evaporation rate, time, and temperature. Coatings with solvent evaporation at ambient conditions (room temperature and atmospheric pressure) typically produced cracked surfaces. Solvent evaporation with heat typically produced fewer cracks than ambient evaporation, but the faster evaporation rate typically produced a more uniform coating. Solvent evaporation with heat and

20   vacuum typically provided reproducibly produced smooth, uniform polymer coatings.

The quality of the polymer coatings was further assessed by an ASTM peel test (ASTM D 3359. West Conshohocken, PA: ASTM International). The results from the qualitative peel test are provided in the following table.

Peel Test Analysis of Coating Stability

Sample	Peel Test Results (Result Code)
PolyAmpicillin (vacuum and heat processing)	Trace peeling along incisions (4A)
PolyAmpicillin (heat only processing)	Jagged removal along most of incisions (3A)

ASTM Peel Test Scale

5A-no peeling or removal

4A-trace peeling or removal

3A-jagged removal along incisions

2A-jagged removal along most of the incisions

1A-removal from most of the area of the X under the tape

0A-removal beyond the area of the X

Peel results showed trace coating removal along x-cut incisions, but overall, the coatings strongly adhere to the stainless steel substrates. These results may be due to a nonspecific chemical interaction between the steel and the coating, ultimately causing the coating to strongly adhere to the stainless steel surface without modification. Upon examination of the heat-processed samples, a jagged removal along the x-cut incisions was noted. Thus, the heat-processed samples typically produce a weaker coating. The vacuum and heat processed samples were typically more robust.

The contact angle of the polymer coatings was about 45°, regardless of the solvent evaporation method. These coatings have intermediate contact angles, which are amenable to degradation, yet not immediately solubilized in aqueous media.

The decomposition temperatures of the polymer coatings prepared by various processing methods were measured by TGA to ensure polymer stability during processing. Polymer (1) displayed a slight increase in the decomposition

temperature following the coating preparation, increasing to a T_d of about 210 °C. Most importantly, during the coating process, the polymer does not decompose.

In Vitro Degradation Study

5   The hydrolytic degradation scheme for the polymer (**1**) is shown below.
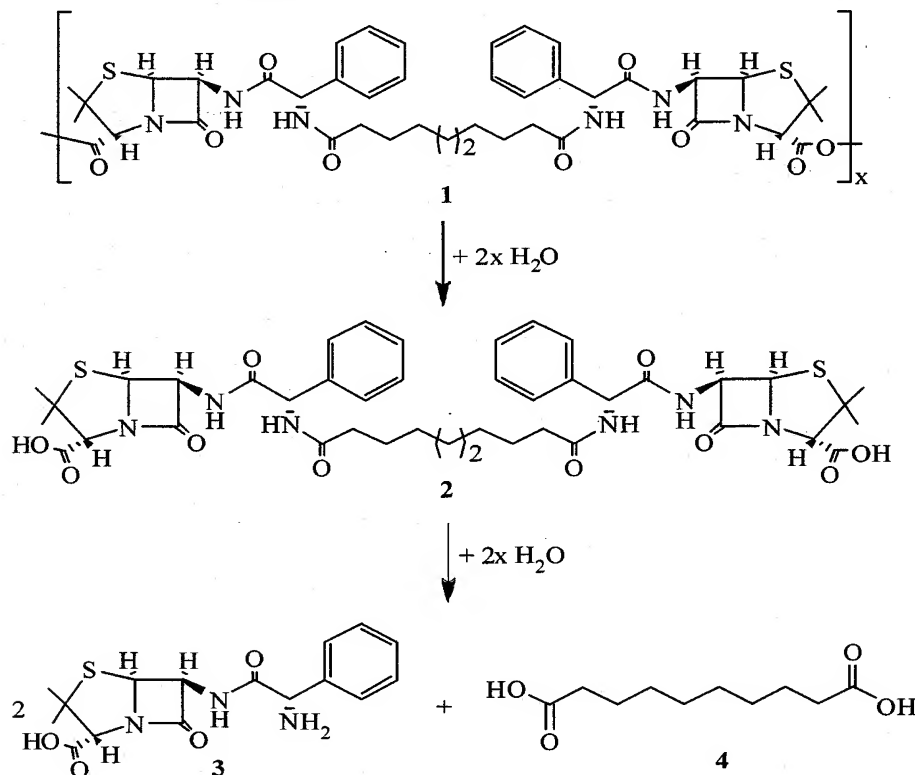
The anhydride bonds are most labile, whereas the amide bonds in the diacid (**2**) intermediate are expected to hydrolyze more slowly and possibly require enzymatic degradation. Initially, the samples were degraded in the SOTAX USP

10   4 Dissolution system, which measures cumulative degradation products with real-time UV detection. Decomposition temperatures as measured by TGA (n=3) for each coating condition are provided below.

Ampicillin-based Polymer Coating Type	Solvent	Solvent Evaporation	T_d (°C)
1 (unprocessed polymer)			195
2	DMF	heat and vacuum	211
3	DMF	heat only	210
4	MeOH	heat and vacuum	208
5	MeOH	heat only	208

Polymer **1** was found to hydrolyze in a more controlled manner with more than 70 % of the ampicillin-based polymer (**1**) coating degraded in 60 hours.

5   Additional release data was obtained from a Varian USP 7 Dissolution Instrument with the ability to separate degradation products by HPLC. In these studies, the free ampicillin drug was not detected because the degradation media was PBS at pH 7.4. The conditions were not amenable to amide bond hydrolysis and there was no ampicillin peak observed in the HPLC chromatogram (results

10   not shown). Again, the diacid intermediate (**2**) was the final degradation product released from the coatings.

Bacterial Adherence Study

   Staphylococcus aureus growth was monitored during exposure to diacid (**2**) and polymer (**1**) coatings, as well as uncoated stainless steel controls. The bacteria

15   cells were seeded to facilitate biofilm growth and were exposed to the coatings for 24 hours. The results of the study are shown in the following table.

Coating (n=3)	Log$_{10}$(Average Cell Count (CFU/mm^2))	± Std. Dev.	Log R	*S. aureus* Biofilm Prevention
Uncoated Control	1.79	0.69	Control	N/A
Polymer 1 (heat and vacuum	2.38	0.04	-0.59	no
Polymer 1 (heat only)	1.71	0.31	0.08	no
Diacid 2	0	0	1.79	yes

The ampicillin-based diacid (2) demonstrated complete prevention of *Staphylococcus aureus* biofilm. In contrast, polymer 1 itself did not inhibit biofilm formation, indicating that the diacid 2 is a bioactive compounds. No

5   significant difference in bacterial growth was observed between the uncoated control and the polymer samples. Given that *Staphylococcus aureus* does not grow on the diacid (2) coating, over longer time periods, it was determined that the diacid will prevent bacteria cell growth and biofilm formation.

Cytotoxicity Assessment

10   Fibroblasts were tested in media containing degraded polymer coatings through heat or through a heat and vacuum methods. Cell proliferation was quantified through 72 hours of culture. Fibroblast culture growth was not impeded by the use of polyampicillin in the cell culture medium. All samples showed positive growth throughout the duration of testing with neither polymer

15   impeding cell proliferation and amplification.

Cell morphology was compared to cells cultured in the control, which contained media without any polymer present. The cells displayed their natural, heterogeneous morphology. Generally, the fibroblasts readily attached and

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remained attached to the surface despite washing and showed characteristic spreading and extensions. However, cells cultured in media containing polymer **1** coatings prepared with fast solvent evaporation (heat and vacuum) did not extend and spread fibers as frequently as the control and polymer **1** coatings

5 prepared with slow solvent evaporation (heat). Nonetheless, cells showed stellate morphology through 72 hours, proving a low cytotoxicity to the polymer degradation products.

Conclusions

Ampicillin-based poly(anhydride-amide) coatings may improve current

10 clinical therapies for bacterial infections. The coatings have strong adhesion properties to 316L stainless steel, which have not been demonstrated in any other polymer system. The *in vitro* degradation studies yielded the ampicillin-based diacid as the final degradation product, in PBS pH 7.4. Although the polymer (**1**) itself was found to be inactive against bacteria growth, it ws found to degrade

15 to the more hydrophilic diacid (**2**), which completely eradicated *Staphylococcus aureus* growth and biofilm formation.

Example 2

Poly(anhydride-amides) that include thermally sensitive β-lactam

20 antibiotics were prepared by solution polymerization. Biodegradable antimicrobial-based polymers in which antimicrobial drugs, namely ampicillin and amoxicillin, were chemically incorporated into the polymeric backbone by anhydride and amide bonds were designed to locally prevent infections associated, *e.g.*, with medical devices, by controlled hydrolytic degradation. The

25 *in vitro* degradation of the polymer into bioactive products was measured and the antibacterial properties examined using gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus aureus*) bacteria. The cytotoxicity of the polymers and bioactive degradation products were screened using fibroblast cells. Overall, the polymer degradation products demonstrated antibacterial

30 activity with no cytotoxic effects on fibroblasts. Based upon these results, the biodegradable antimicrobial-based poly(anhydride-amides) display promise as

implants or implant coatings to reduce implant failure resulting from bacterial infections.

Results and Discussion

        Polymer Synthesis and Physicochemical Characterization. Based on

5   the functional groups present in the antimicrobial molecules (**1**), the compounds were chemically incorporated into the polymer backbone through the more nucleophilic primary amine groups, whereas the carboxylic acids were used to ultimately generate the anhydride linkages as outlined in the scheme below.
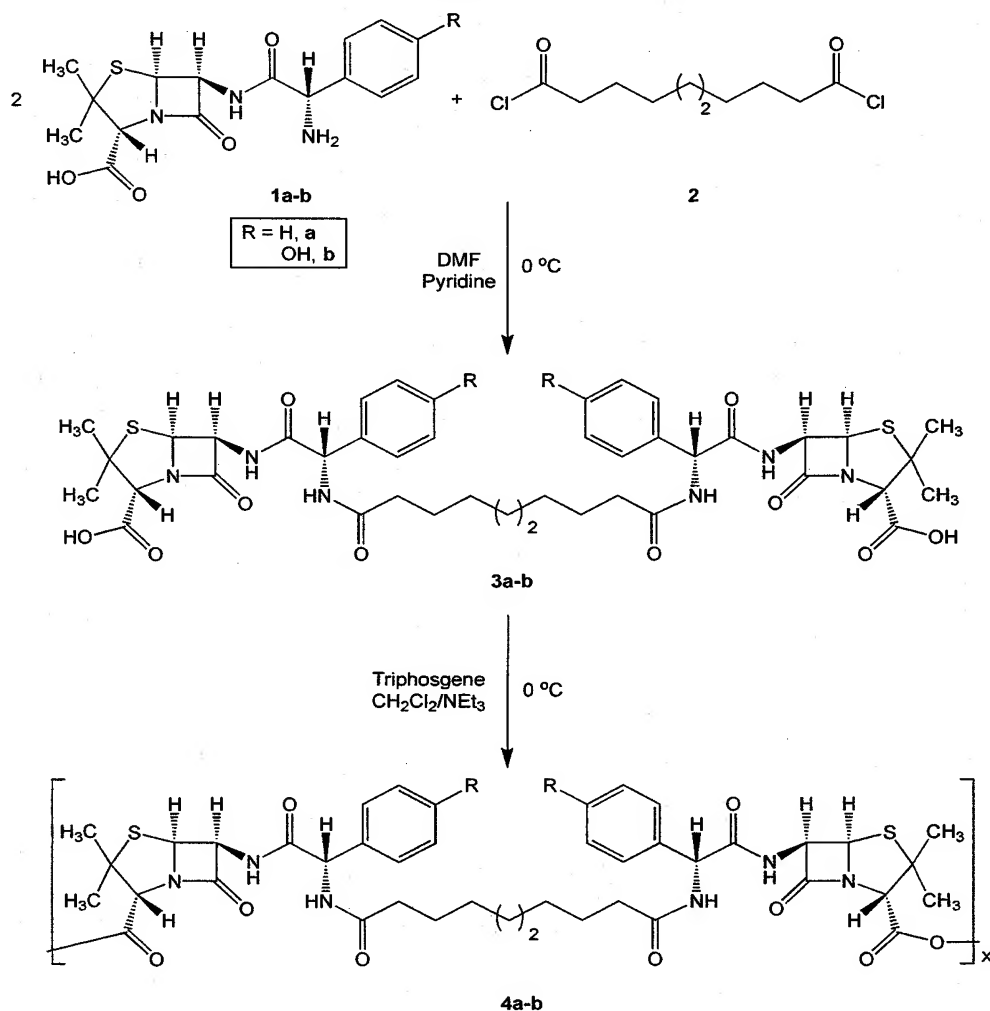
10

Synthesis of antimicrobial-based poly(anhydride-amides), **4** comprised of either ampicillin (**a**) or amoxicillin (**b**).

        The polymer precursor or diacid (**3**) was synthesized by direct coupling of the antibiotic (**1**) (ampicillin, **a**, or amoxicillin, **b**) with sebacoyl chloride (**2**)

in dimethylformamide (DMF) containing pyridine. At low reaction
temperatures, the primary amino group of the antibiotic reacts with the acyl
chlorides of sebacoyl chloride (**2**) to form **3** in reasonable yields. In this
conversion, pyridine acts as acid acceptor by neutralizing the HCl by-product.

5   The ^{1}H and ^{13}C NMR spectra clearly indicate that the pure products are obtained.
The potential for side reactions, particularly the phenol of amoxicillin to also
react with the sebacoyl chloride, was a concern yet only the expected product
(**4b**) was isolated and used in the polymerization reactions.

With the thermal instability of the antibiotics (**1**) and antimicrobial-based

10   diacids (**3**), the polymers could not be generated *via* melt-condensation
conditions typically used to generate drug-containing polyanhydrides. Therefore,
the diacids were polymerized under mild conditions in methylene chloride at 0
°C using triphosgene (1/3 equivalents) as a coupling agent and triethylamine as
acid acceptor to form the poly(anhydride-amide) (**4**) in nearly quantitative yields.

15   The chemical incorporation of the β-lactam antibiotics, ampicillin **1a** and
amoxicillin **1b**, into polymeric backbones yielded polymers with drug loadings
of nearly 82 % by weight. Molecular weights of the poly(anhydride-amides)
synthesized from these antimicrobials ranged from 14,400-24,800 Da. These
moderate molecular weights are similar to other poly(anhydride-ester) molecular

20   weights obtained by melt-polymerization methods. Polymers 4 displayed no
glass transition temperatures (T_g) from −10 °C to the temperatures just below
decomposition (~200 °C). The ampicillin-based poly(anhydride-amide) (**4a**)
displayed a higher decomposition temperature (T_d) of 235 °C than the
amoxicillin-based polymer (**4b**) with T_d 190 °C. Notably, the antimicrobial-

25   containing polymers (**4**) were relatively more soluble in organic solvents than the
free antibiotic (**1**); this characteristic is particularly significant for polymer
processability.

In Vitro **Degradation.** Hydrolytic degradation of the poly(anhydride-
amides) was measured on polymer disks immersed in PBS at 37 °C for up to 3

30   days. In these experiments, only the polymer degradation intermediate (diacid **3**)
was observed in the degradation media; the free antibiotic (**1**) was not detected.
After two days of *in vitro* incubation, the anhydride linkages of the polymers (**4**)

are completely hydrolyzed to yield the intermediate **3**. However, no amide
cleavage to release the free antibiotic (**1**) was observed *in vitro*; hydrolysis of
amides typically requires amidases or extreme pH conditions.

Additional ¹H NMR studies confirmed this observation, in which diacids
5   (**3**) were dissolved in DMSO/PBS and monitored. Hydrolytic cleavage of the
amide bonds of **3** to release the free antibiotic (**1**) and sebacic acid was
investigated by incubating the diacids (**3**) at 37 °C for three days. The presence
of free antibiotic (**1**) was not detected by NMR spectroscopy, indicating that the
amide bond is stable under these conditions.

10   **Antimicrobial Assessment.** The Kirby-Bauer or disk diffusion method
is a relatively simple and reliable method used to test antimicrobial susceptibility
for drugs and provides data that can reliably predict the effectiveness of the
compound *in vivo* (Antibacterial susceptibility tests: dilution and disk diffusion
methods. In Manual of Clinical Microbiology; Murray, P.R., Pfaller, M.A.,
15   Tenover, F.C., Baron, E.J.,Yolken, R.H., Eds.; ASM Press: Washington DC,
1999; 1526-1543; Performance standards for antimicrobial susceptibility testing.
In National Comittee for Clinical Laboratory Standards (NCCLS); Eds.;
document M100-S9: Wayne, PA, 1999; 2I). The antimicrobial activity of the
poly(anhydride-amides) as well as their degradation products against *Escherichia*
20   *coli* and *Staphylococcus aureus* was investigated *in vitro* using the disk diffusion
method. Paper disks impregnated with test solutions were placed on agar plates
uniformly inoculated with either *E. coli* or *S. aureus*. As the compounds
diffused out of the disk over a 24 h incubation period at 37 °C, a circular zone of
inhibition was observed around the disk indicating that the compound may be
25   either bactericidal or bacteriostatic. Antibacterial efficiency was assessed by
measuring the zone of inhibition surrounding the polymer-impregnated disk.
The diameters of the inhibition zones are reported in the Table below against *S.*
aureus and *E. coli* strains.

Table. Zones of inhibition (mm) of test compounds against *Escherichia coli*, and *Staphylococcus aureus*. Inhibition zones were not observed for controls (media only).

Compound	Inhibition Zone Diameter (mm)	
	E. coli	*S. aureus*
Amoxicillin (**1b**)	23	55
Amoxicillin-based Diacid (**3b**)	11	31
Amoxicillin-based Polymer (**4b**)	-	21
Ampicillin (**1a**)	26	58
Ampicillin-based Diacid (**3a**)	15	34
Ampicillin-based Polymer (**4a**)	10	22

5

Among the bacteria tested, *S. aureus* (gram-positive) was the most susceptible organism; test compounds showed more potent activity against gram-positive than gram-negative bacteria. The slightly smaller inhibition zones on the plates containing *E. coli* compared to those with *S. aureus* reflects the

10   difficulty for the test compounds to diffuse across the extra outer membrane present in the gram-negative *E. coli*.

Unexpectedly, the diacids **3** themselves exhibited an inhibitory effect on the bacteria, displaying inhibition zones with diameters in the range of 10-15 mm for *E. coli* and 31-34 mm for *S. aureus*. This phenomenon is notable because the

15   NMR and *in vitro* degradation studies indicated that free antibiotic (**1**) was not formed from diacid **3** in media alone. Inhibition zones were not necessarily expected for the polymers (**4**), given that their large size may not allow for diffusion through the bacterial membrane. However, small inhibition zones were observed surrounding the polymer samples. These results indicate that the

20   hydrolysis of the polymer (**4**) into the diacids (**3**) is sufficient to generate inhibitory concentration of diacid.

Cytotoxicity Assessment. The cytotoxicity of the polymers **4** and their degradation products were evaluated *in vitro* with L929 fibroblasts, the most predominant tissue cells in the body and the standard cells recommended by the

American Society for Testing and Materials (ASTM) in testing materials. For this study, polymer solutions of 0.1 mg/mL and 0.01 mg/mL were prepared in DMSO/media, then fibroblast morphology and proliferation evaluated. The range of polymer concentrations was chosen based on polymer cytotoxicity test

5   protocols developed by the Uhrich laboratory (Schmalenberg, K., Frauchiger, L., Nikkhouy-Albers, L., Uhrich, K. Biomacromolecules 2001, 2, 851-855; Weber, N., Wendel, H., Kohn, J. J. Biomed. Mater. Res. 2005, 72A, 42-47; Sharma, R., Kohn, J., Moghe, P. J. Biomed. Mater. Res. 2004, 69A, 114-123). Solutions containing media and DMSO at the same concentrations used to prepare the

10   polymer solutions were used as controls. Cellular morphologies were compared to the control sample every 24 h over a 3-day period. In general, no significant morphological changes were observed for cells in polymer solutions of 0.01 mg/mL and 0.1 mg/mL relative to the controls of DMSO/media and media alone. For both 0.01 and 0.1 mg/mL polymer concentrations, the cells were attached

15   onto the surface of the plate and grew with normal stellate morphology over the 3 days. Similarly to the controls of DMSO/media and media alone, high density of cells is observed by the end of the 3-day period. In this study, round appearance of cells is not an indication of cell death but of a high cell density as confirmed by live cell counts.

20   Based on the *in vitro* degradation experiments described herein, the 3-day cell cultivation time was sufficient to guarantee cell contact with the polymers and to allow complete cell cycles. Cells exhibited positive growth cycles in all the polymer solutions at every concentration and no statistical significance was observed for all polymers with respect to the control (Student's *t*-test, 95%). In

25   general, polymers **4** showed good results at both polymer concentrations with respect to cell proliferation/cytotoxicity.

Materials. Ampicillin and amoxicillin were obtained from MP Biomedicals (Solon, OH). Dimethylformamide (DMF), dimethylacetamide (DMA, anhydrous), pyridine, triethylamine, acetic anhydride, methylene chloride

30   (anhydrous), and diethyl ether were purchased from Fisher (Fair Lawn, NJ). All other fine chemicals and solvents were obtained from Aldrich (Milwaukee, WI). Triethylamine was dried over calcium hydride (CaH_2) and all other reagents

were used with no further purification.

Methods and Equipment. Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on a Varian 300 MHz spectrometer. The samples (5-10 mg) were dissolved in a deuterated solvent (DMSO-d_6), which was also
5   used as the internal reference. Infrared (IR) spectra were measured on a Thermo Nicolet/Avatar 360 FT IR spectrometer, by solvent-casting samples from methylene chloride or methanol onto NaCl plates (if solid).

Weight-averaged molecular weights (M_w) and polydispersity indices (PDI) were determined by gel permeation chromatography (GPC) on a Waters
10   liquid chromatography system consisting of a Waters 410 refractive index detector, a Waters 510 LC pump, and an ISS 200 advanced sample processor. An IBM Thinkcenter computer running Waters Empower software was used for data collection and processing. Polymers were dissolved (5 mg/mL) in dimethylformamide (DMF) containing 0.1 trifluoroacetic acid (TFA) and filtered
15   through 0.45 μm poly(tetrafluoroethylene) (PTFE) syringe filters (Whatman, Clifton, NJ) before elution. Samples were resolved on two mixed-bed GPC columns (Polymer Laboratories, Amherst, MA) (Alltech Associates, Deerfield, IL) placed in series at 25 °C, with DMF containing 0.1% TFA as eluent at a flow rate of 0.8 mL/min. Molecular weights were calibrated relative to narrow
20   molecular weight polystyrene standards (Polysciences, Dorval, Canada).

Thermal analyses were performed on a Perkin-Elmer system consisting of Pyris 1 DSC and TGA 7 analyzers with TAC 7/DX instrument controllers. Perkin-Elmer Pyris software was used for data collection on a Dell OptiPlex GX110 computer. For DSC, samples (5 mg) were heated under dry nitrogen gas.
25   Data were collected at heating and cooling rates of 10 °C/min with a two-cycle minimum, starting from −10 °C to just below decomposition (~200 °C). Glass transition temperatures were calculated as half Cp extrapolated. For TGA, samples (10 mg) were heated under dry nitrogen gas. Data were collected at a heating rate of 10 °C/min. Decomposition temperatures were defined as the
30   onset of decomposition.

Elemental analyses were provided by QTI (Whitehouse, NJ). Melting points were obtained with a Mel-Temp apparatus at a heating rate of 1 °C per minute.

5   Sessile-drop contact angles of phosphate buffer solution on polymer disks were measured using an automated Ramé-hart goniometer (Model 250, Netcong, NJ) with DROPimage Advanced software on a Dell Dimension 3000 computer. Angles were measured on three different disks for each polymer and an averaged value was taken.

Poly(anhydride-amide) Precursors: Diacid Synthesis (3). Diacids (3)
10   were prepared by reaction of the appropriate antimicrobial **1** with sebacoyl chloride (**2**) in the presence of a base (pyridine). The preparation of **3a** is provided as an example. Ampicillin (**1a**) (3.5 g, 10 mmol) was added to DMF (100 mL) and pyridine (4.0 mL, 50 mmol) to afford a suspension. Sebacoyl chloride (**2**) (1.1 mL, 5.2 mmol) dissolved in dimethylformamide (10 mL) was
15   added dropwise over 5 minutes to the stirring reaction mixture at 0 °C to afford a clear solution. The reaction was stirred for 2 h at 0 °C, poured over water (400 mL) and acidified to pH~2 using 0.1 N hydrochloric acid solution while stirring. The white solid (diacid, **3**) that formed was isolated by vacuum filtration, washed with water (3x100 mL) and dried overnight under vacuum at room
20   temperature.

Ampicillin-sebacic Diacid (3a). Yield: 82% (white powder). ^{1}H NMR (DMSO-d_6): δ 9.10 (d, 2H, NH), 8.50 (d, 2H, NH), 7.40 (m, 10H, ArH), 5.75 (d, 2H, CH), 5.55 (m, 2H, CH), 5.40 (d, 2H, CH), 4.20 (s, 2H, CH), 2.25 (t, 4H, CH$_2$), 1.65-1.35 (m, 12H, CH$_2$), 1.20 (s, 12H, CH$_3$). ^{13}C-NMR (DMSO-d_6): δ
25   173.7, 172.3, 170.5, 169.1, 138.6, 128.4, 127.8, 127.4, 70.6, 67.5, 64.0, 58.5, 55.7, 35.2, 30.7, 29.0, 26.9, 25.6IR (NaCl, cm^{-1}): 3500-3100 (OH, COOH), 1785 (C=O, COOH), 1730 (C=O, amide I), 1645 (C=O, amide I), 1645 (C=O, amide I), 1535 (NH, amide II), 1510 (NH, amide II). Anal. Calcd.: C, 58.3%; H, 6.1%; N, 9.7%; S, 7.4%. Found: C, 57.7%; H, 6.3%; N, 9.5%; S, 7.4%. T_d = 200 °C.

30   **Amoxicillin-sebacic Diacid (3b).** Yield: 48% (white powder). ^{1}H NMR (DMSO-d_6): δ 8.90 (d, 2H, NH), 8.30 (d, 2H, NH), 7.10 (d, 4H, ArH), 6.65 (d, 4H, ArH), 5.50 (m, 4H, CH), 5.35 (d, 2H, CH), 4.20 (s, 2H, CH), 2.15 (t, 4H,

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CH$_2$), 1.60-1.35 (m, 12H, CH$_2$), 1.15 (s, 12H, CH$_3$). ^{13}C-NMR (DMSO-d$_6$): δ
173.9, 172.2, 171.0, 169.2, 157.0, 128.7, 128.7, 115.2, 70.6, 67.5, 64.0, 58.3,
55.3, 35.2, 30.6, 29.0, 26.9, 25.6 IR (NaCl, cm^{-1}): 3550-3100 (OH, COOH),
1775 (C=O, COOH), 1730 (C=O, amide I), 1660 (C=O, amide I), 1645 (C=O,
amide I), 1550 (NH, amide II), 1515 (NH, amide II). Anal. Calcd.: C, 56.2%; H,
5.8%; N, 9.3%; O, 22.0%; S, 6.7%. Found: C, 55.8%; H, 6.2%; N, 8.7%; O,
22.2%; S, 7.1%. T$_d$ = 173 °C.

Polymer Synthesis (4). Diacid (3) (0.30 mmol) was dissolved 20 %
(w/v) methylene chloride and triethylamine (1.3 mmol). Triphosgene (0.33
mmol) dissolved in methylene chloride (5 mL) was added dropwise at a rate of 1
drop/4 sec to the stirring reaction mixture at 0 °C to afford a suspension. The
reaction was stirred for 2 h at 0 °C under nitrogen and poured over diethyl ether
(100 mL). The solid (polymer 4) that formed was isolated by vacuum filtration,
washed with acidic water (3x100 mL) and dried overnight under vacuum at room
temperature. Any changes to the synthetic procedure are noted below.
Polymers 4 are soluble in organic solvents such as methanol,
dimethylformamide, and dimethylsulfoxide.

Poly[ampicillin-sebacic] (4a). Yield: quantitative (yellow powder). ^{1}H
NMR (DMSO-d$_6$): δ 9.10 (d, 2H, NH), 8.45 (d, 2H, NH), 7.40 (m, 10H, ArH),
5.75 (d, 2H, CH), 5.55 (m, 2H, CH), 5.35 (d, 2H, CH), 4.20 (s, 2H, CH), 2.20 (t,
4H, CH$_2$), 1.60-1.30 (m, 12H, CH$_2$), 1.15 (s, 12H, CH$_3$). IR (NaCl, cm^{-1}): 1795,
1760 (C=O, anhydride), 1730 (C=O, amide I), 1650 (C=O, amide I), 1605 (C=O,
amide I), 1540 (NH, amide II), 1515 (NH, amide II). M$_w$ = 24800, PDI = 1.8. T$_d$
= 235 °C. Contact angle: 27 °.

Poly[amoxicillin-sebacic] (4b). Dichloromethane:dimethylacetamide
(1:1) was used as solvent. Yield: quantitative (orange powder). ^{1}H NMR
(DMSO-d$_6$): δ 8.90 (d, 2H, NH), 8.30 (d, 2H, NH), 7.10 (d, 4H, ArH), 6.65 (d,
4H, ArH), 5.55 (m, 4H, CH), 5.35 (d, 2H, CH), 4.15 (s, 2H, CH), 2.15 (t, 4H,
CH$_2$), 1.55-1.30 (m, 12H, CH$_2$), 1.15 (s, 12H, CH$_3$). IR (NaCl, cm^{-1}): 1800,
1760 (C=O, anhydride), 1720 (C=O, amide I), 1705 (C=O, amide I), 1645 (C=O,
amide I), 1575 (NH, amide II), 1515 (NH, amide II). M$_w$ = 14400, PDI = 1.2. T$_d$
= 190 °C. Contact angle: 32 °.

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In Vitro **Degradation: Sample and Media Preparation.** Polymer pellets were prepared by pressing ground polymers (14.0 ± 0.5 mg) into 6 mm diameter x 0.5 mm thick disks in an IR pellet die (International Crystal Laboratories, Garfield, NJ) with a bench-top hydraulic press (Carver model M, Wabash, IN).

5   A pressure of 5,000 psi was applied for 5 min at room temperature. No change in polymer color was observed upon applying pressure.

Degradation media consisted of phosphate buffer solution (PBS) containing 0.1 M potassium hydrogen phosphate and 0.1 M potassium dihydrogen phosphate. The pH was adjusted to 7.4 with 1 M sodium hydroxide

10   and/or 1 N hydrochloric acid solutions and pH-measurements were performed on an Accumet® AR15 pH-meter (Fisher Scientific, Fair Lawn, NJ).

In Vitro **Degradation: Analysis.** Hydrolytic degradation of polymers **4** was performed by placing the disks in 20 mL Wheaton glass scintillation vials (Fisher, Fair Lawn, NJ) with 10 mL of PBS, and incubating them at 37 °C with

15   agitation using a controlled environment incubator-shaker (New Brunswick Scientific Co., Edison, NJ) at 60 rpm for 3 days. The buffer solution was replaced by fresh solution (10 mL) every 24 h. The spent media was analyzed by UV spectroscopy at each time point.

Diacid **3** concentration in the degradation media at each time point was

20   determined by UV spectroscopy at λ = 209 nm (**3a**) and 206 nm (**3b**). UV data were calibrated against standard solutions of known concentrations. Data (average of 3 samples per time point) were presented as a cumulative percent over time.

To further evaluate diacid **3** degradation into the corresponding

25   antimicrobials (**1**), diacids were dissolved in deuterated dimethylsulfoxide (DMSO-d_6) and PBS (pH = 7.4), at 4:1 (v/v) and incubated at 37 °C over 3 days. Every 24 h, media was analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy for the appearance of free antimicrobial (**1**).

Antimicrobial Assays: Polymers and Bacterial Strain. The inhibition

30   zone test of polymers and their degradation products against the bacteria *Escherichia coli* and *Staphylococcus aureus* was carried out according to the standardized protocol approved by the National Committee for Clinical

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Laboratory Standards (NCCLS) (Antibacterial susceptibility tests: dilution and
disk diffusion methods. In Manual of Clinical Microbiology; Murray, P.R.,
Pfaller, M.A., Tenover, F.C., Baron, E.J.,Yolken, R.H., Eds.; ASM Press:
Washington DC, 1999; 1526-1543; Performance standards for antimicrobial
5   susceptibility testing. In National Comittee for Clinical Laboratory Standards
(NCCLS); Eds.; document M100-S9: Wayne, PA, 1999; 2I).

Stock solutions of all polymers (4), diacids (3) and antimicrobials (1) (10
mg/mL) were prepared in dimethylsulfoxide (DMSO) and then diluted in PBS at
1:4 (v/v) to give final concentrations of 2.0 mg/mL. A control containing
10   DMSO/PBS was utilized to negate any antibacterial effect due to DMSO.

Two bacterial strains *Escherichia coli B* and *Staphylococcus aureus* were
used. Bacteria inocula were suspended in nutrient broth (EMD Chemicals,
Gibbstwon, NJ) and culture turbidity adjusted to match that of a 0.5 McFarland
standard (see below) to give a bacterial count of approximately 10^8 colony
15   forming units/mL.

Antimicrobial Assays. McFarland 0.5 turbidity standard was prepared
according to the protocol described by the National Committee for Clinical
Laboratory Standards (NCCLS) (Performance standards for antimicrobial
susceptibility testing. In National Comittee for Clinical Laboratory Standards
20   (NCCLS); Eds.; document M100-S9: Wayne, PA, 1999; 2I). Briefly, 0.5 mL of
a 1.175% (wt/v) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) (Aldrich, Milwaukee,
WI) solution was added to 99.5 mL of 1% (v/v) sulfuric acid (Fisher, Fair Lawn,
NJ). The McFarland standard was stored at room temperature in the dark.

Mueller-Hinton II agar nutrient medium (Becton Dickinson, Sparks, MD)
25   was prepared according to manufacturer's instructions. Briefly, agar (38 g) was
suspended in 1 L of deionized water and heated to boil with agitation to
complete dissolution. Solution was autoclaved (Amsco Scientific Series 3021-S,
Apex, NC) at 121 °C for 20 min and the sterile medium (pH = 7.2-7.4, 25 mL)
poured at 50-60 °C onto 15 x 100 mm sterile petri dishes (Fisher, Fair Lawn, NJ)
30   to an uniform thickness of 4 mm. Freshly prepared plates were refrigerated at 2 °
to 8 °C before use.

Antimicrobial Assays: Disk Diffusion Method. The Mueller-Hinton II agar plate surface was inoculated with the broth culture of *Escherichia coli* and *Staphylococcus aureus* using a cotton swab (Fisher, Fair Lawn, NJ). Sterile paper disks (6 mm-diameter, Becton Dickinson, Sparks, MD) impregnated with 20 μL of the test solutions were placed and gently pressed down onto the plates using sterile forceps. Only four disks were placed on each plate to minimize overlap of the inhibition zones. The plates were incubated (Precision Scientific Incubator, South Plainfield, NJ) at 37 °C for 24 h under aerobic conditions. After incubation, the plates were examined and inhibition zones surrounding the disks were measured in millimeters using a ruler. All determinations were carried out in triplicate.

Cytotoxicity Studies: Polymer Samples. Cytotoxicity of the polymers was analyzed by culturing cells in media containing the polymer. To prepare the polymer stock solution, the polymers were first sterilized by UV irradiation for 300 s and dissolved in DMSO to 40 mg/mL. The stock solution was then diluted with cell culture medium to three different final concentrations (1, 0.1 and 0.01 mg/mL). Cell culture media consisted of Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO) with 10 % v/v fetal bovine serum, 1 % v/v glutamine, and 50 U/mL of penicillin and streptomycin. Media containing DMSO at the concentration used to prepare the polymer solutions was used as control.

Cytotoxicity Studies: Fibroblast Cultures. L929 mouse areolar/adipose fibroblasts (Department of Biomedical Engineering, Rutgers University, Piscataway, NJ) were incubated in cell culture medium at 37 °C in a humidified atmosphere (5 % CO_2). Confluent fibroblasts were detached by trypsin (0.02 mg/mL, Sigma, St. Louis, MO) and incubated at 37 °C for 5 min. Trypsin activity was stopped by adding cold media, cell pellets were obtained by centrifugation (Thermo Electron 5682 3L GP, Franklin, MA) at 2000 rpm for 2 min. Cells were seeded onto the wells of a 24-well plate containing the polymer solutions at a density of 5×10^4 cells per well and incubated for 3 days. All experiments were performed in triplicate for each polymer solution.

Cytotoxicity Studies: Cell Morphology and Proliferation. Fibroblast morphology in each solution was observed using a fluorescent microscope

(IX81, Olympus, Japan) equipped with 10X phase-contrast objective at days 1, 2 and 3.

Cell growth in each solution was evaluated every 24 h, after imaging. Cell numbers were measured using Calcein AM staining as a live-dead assay. Calcein AM solution is a cell membrane permeable fluorescent marker only for live cell cytoplasms (Cutler, S., Garcia, A. Biomaterials 2003, 24, 1759-1770). At each time point, medium was removed and fibroblasts were gently washed twice with PBS (MP Biomedical, Aurora, OH) then incubated with 200 µL of Calcein AM staining solution (8 µM, Molecular Probe, Carlsbad, CA) for 40 min at 4 °C. Fluorescent intensity was measured using a fluorescence plate reader (Cytofluor® Series 4000, Applied Biosystems, Woodinville, CA) at 485 nm excitation and 530 nm emission. Live cell numbers were calculated against a standard curve. All polymers were assayed in triplicate.

Fibroblast growth in polymer solutions was statistically compared to controls at each time point in triplicate. Student's t-test was performed using Microsoft EXCEL® software at an accepted significance of $p < 0.05$.

All publications, patents, and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

CLAIMS

What is claimed is:

1.   A compound of formula II:
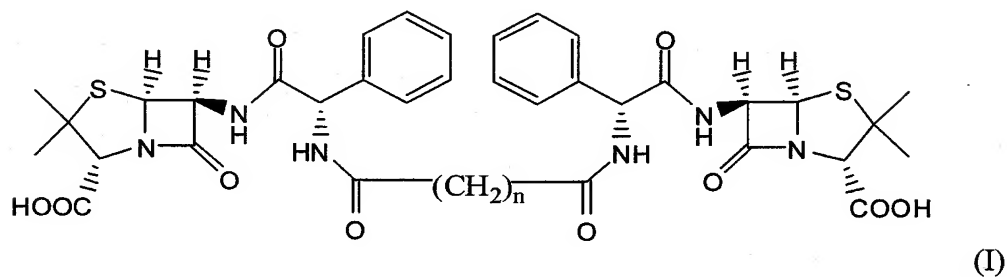

(II)
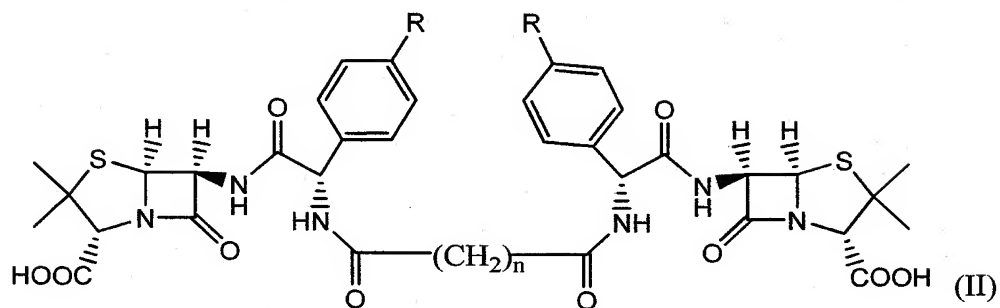
wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12; and

each R is independently H or OH;

or a salt thereof.

2.   The compound of claim 1, wherein each R is OH.

3.   A compound of formula I:

(I)

wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12; or a salt thereof.

4.   The compound of any one of claims 1-3, wherein n is 4, 5, 6, 7, or 8.

5.   The compound of claim 4, wherein n is 6, 7, or 8.

6.   The compound of claim 5, wherein n is 8.

7.   A pharmaceutical composition comprising a compound of formula I or II as described in any one of claims 1-6; or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable diluent or carrier.

8.   A medical device having a coating that comprises a compound of formula I or II as described in any one of claims 1-6; or a pharmaceutically acceptable salt thereof.

9.   A medical device comprising a compound of formula I or II as described in any one of claims 1-6; or a pharmaceutically acceptable salt thereof.

10.   The medical device of claim 8 or 9, which is an orthopedic implant.

11.   The medical device of claim 8 or 9, which is a knee joint or a hip joint.

12.   A method to promote an anti-bacterial effect in an animal comprising administering a compound of formula I or II as described in any one of claims 1-6; or a pharmaceutically acceptable salt thereof to the animal.

13.   A method to promote an antibacterial effect comprising contacting bacteria with a compound of formula I or II as described in any one of claims 1-6, or with a salt thereof.

14.   A method to reduce biofilm formation on a surface comprising contacting the surface with a compound of formula I or II as described in any one of claims 1-6; or a salt thereof.

15.   The method of claim 14, wherein the compound of formula I or II or the salt thereof is incorporated into a coating that is on the surface.

16.   A compound of formula I or II as described in any one of claims 1-6, or a pharmaceutically acceptable salt thereof for use in medical therapy.

17.   The use of a compound of formula I or II as described in any one of claims 1-6, or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for producing an anti-bacterial effect in a mammal.

18.   The compound of formula I or II as described in any one of claims 1-6; or a pharmaceutically acceptable salt thereof, for the prophylactic or therapeutic treatment of a bacterial infection.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/74070

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 43/00; A61K 31/397; A61K 31/54 (2008.04)
USPC - 514/210.01; 514/226.8
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 514/210.01; 514/226.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/195; 514/210.01; 514/210.03; 540/330; 540/331 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST (PGPB,USPT,EPAB,JPAB): ampicillin, amoxicillin, polyanhydride, amide
esp@cenet: ampicillin, amoxicillin, uhrich, rutgers
Google Scholar: ampicillin, amoxicillin, polyanhydride

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/0013851 A1 (GIROUX) 19 January 2006 (19.01.2006) pg 9-11	1-6
A	US 2005/0100526 A1 (UHRICH) 12 May 2005 (12.05.2005)	1-6

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2008 (11.12.2008)	18 DEC 2008

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2007)

| Box No. II | Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet) |

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box No. III | Observations where unity of invention is lacking (Continuation of item 3 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (April 2007)